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A new application of the SFDA-staining method to assessment of the freezing tolerance in leaves of alpine plants

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Abstract: For the first time, this study used 5- (6-) sulfofluorescein diacetate (SFDA), a fluorescent product in plant cells converted by esterase activity to fluorescein-5- (and 6-) sulfonic acid (FSA), to assess the freezing tolerance of leaf cells. We were able to readily distinguish living and dead cells, and detect differences in freezing tolerance among five alpine plants using the SFDA-staining method. We also compared this method with two conventional methods, the electrolyte leakage test and fluorescein diacetate (FDA) staining method. The electrolyte leakage test often over- or underestimated freezing injury. With the uninjured control samples, the FDA-staining method failed to stain all leaf cells, while the SFDA-staining method stained almost 100%. From these results, we concluded that SFDA-staining is a more convenient, accurate and reproducible method for analyses of freezing tolerance.

key words: alpine plants, FDA (fluorescein diacetate), freezing tolerance, SFDA (5- (6-) sulfofluorescein diacetate), survival ratio

Introduction

Examining cell viability is useful in many physiological, biotechnological and ecological studies. For example, according to Steponkus and Lanphear (1967), a detailed description of freezing injuries and cold acclimation in plants is necessary for accurately determining the extent of injury. Various methods have been developed to conduct viability tests, with the most commonly used including the regrowth capacity and tissue browning test (Sakai, 1955; Neuner *et al*., 1997; Chen *et al*., 2000; Menges and Murray, 2004; Wang *et al*., 2004), the electrolyte leakage test (Dexter, 1956; Hurry *et al*., 2000; Pennycooke *et al*., 2003; Shou *et al*., 2004; Yamori *et al*., 2005), the vital staining method (Widholm, 1972; Smith *et al*., 1982; Huang *et al*., 1986; Swain and De, 1994; Hou and Lin, 1996), the 2, 3, 5-triphenyltetrazolium chloride (TTC) reduction test (Steponkus and Lanphear, 1967; Towill and Mazur, 1974; Ishikawa *et al*., 1995; Pihakaski-Maunsbach *et al*., 2003; Wang *et al*., 2004) and the fluorescein diacetate (FDA) staining

Table 1. A summary of the main viability tests currently employed, including their advantages and disadvantages comments.

Method	Comments
Regrowth capacity test	(Advantages)
Tissue browning test	Currently, the most reliable viability tests (1, 2, 3, 4, 5).
	Able to evaluate differences in stress tolerance among different plant tissues, including recovery following stress (3, 4).
	(Disadvantages)
	Must be conducted under aseptic conditions, and the incubation period is time-consuming (2, 4, 5).
Electrolyte leakage test	(Advantages)
	Able to rapidly evaluate cell injury in many samples (3, 6, 7).
	(Disadvantages)
	Tends to overestimate and/or underestimate the injury (3, 8, 9, 10, 11, 12).
	Not able to estimate comprising tissues differing in their freezing tolerance capacity (13, 14, 15).
Vital staining method	Includes the staining methods of evans blue (16, 17, 18), trypan blue (19), neutral red (18, 20, 21), methylene blue (22), and phenosafranine (18, 23).
	(Advantages)
	Rapid and needs only a light microscope for observation (23).
	(Disadvantages)
	Needs laborious and inaccurate microscopic observations in the case of natural aggregation of plant cells (24).
TTC reduction test	(Advantages)
	Does not diffuse out of cells after reduction (25).
	Rapid and convenient (5, 25, 26, 27, 28).
	(Disadvantages)
	Needs laborious and inaccurate microscopic observations in the case of natural aggregation of plant cells (5, 25, 26, 29).
	Depends not only on the cell viability but also on the metabolic state of the cells, such as the amount of reducing sugars, glutathione, ascorbic acid, and cysteine (25, 30, 31, 32).
	Probably not considered a general test for cell survival (4).
FDA-staining method	(Advantages)
	Convenient, simple and reproducible in cultured cells and monads (5, 23, 24), roots and seeds (33) and tissue sections of higher plants (12).
	(Disadvantages)
	Tends to underestimate injury (12, 24, 34, 35, 36).
	Cannot be applied to comprised cells (5, 37).

(1) Sakai, 1955; (2) Sterigo and Howell, 1973; (3) Burr *et al.*, 1990; (4) James and Bert, 1990; (5) Ishikawa *et al.*, 1995; (6) Dexter *et al.*, 1930; (7) Dexter *et al.*, 1932; (8) Ketche *et al.*, 1972; (9) Van den Driessche, 1976; (10) Pellett and Carter, 1981; (11) Boorse *et al.*, 1998; (12) Yamori *et al.*, 2005; (13) Dexter, 1956; (14) Aronsson and Eliasson, 1970; (15) Iija and Jiri, 1998; (16) Gaff and Okong'Oogola, 1971; (17) Smith *et al.*, 1982; (18) Swain and De, 1994; (19) Hou and Lin, 1996; (20) Nobel *et al.*, 1995; (21) Nobel and Barrera, 2003; (22) Huang *et al.*, 1986; (23) Widholm, 1972; (24) Steward *et al.*, 1999; (25) Parker, 1953; (26) Towill and Mazur, 1974; (27) Duncan and Widholm, 1990; (28) Burke, 2001; (29) Ishikawa *et al.*, 1990; (30) Mattson *et al.*, 1947; (31) Nash and Davies, 1975; (32) Matanguihan *et al.*, 1994; (33) Noland and Mohammed, 1997; (34) Ogawa *et al.*, 1997; (35) Wang *et al.*, 2001; (36) Winkelmann *et al.*, 2004; (37) Harding and Benson, 1995.

method (Widholm, 1972; Vaňková *et al.*, 2001; Pokorna *et al.*, 2004; Winkelmann *et al.*, 2004; Yamori *et al.*, 2005) (Table 1). Each method has a number of advantages and disadvantages (Table 1), and there is no single viability test that can be used for all types of plant material or under all types of experimental conditions.

The fluorescein diacetate (FDA) staining method can be reliably used to determine plant cell survival using cultured cells and monads (Widholm, 1972; Ishikawa *et al.*, 1995; Steward *et al.*, 1999). FDA passing through the cell membrane is converted into a fluorescent substance, fluorescein, by endogenous esterase activity. In general, it is possible to distinguish living from dead cells using this method. However, FDA does not efficiently penetrate some types of membrane in certain species (Harding and Benson, 1995). Moreover, the product resulting from the conversion of FDA, fluorescein, tends to leak from the cells (Söderstöme, 1979; Thomas *et al.*, 1979). Previous studies have shown that the method fails to give 100% cell survival ratios even for uninjured control samples of cultured cells (Ogawa *et al.*, 1997; Steward *et al.*, 1999; Wang *et al.*, 2001; Winkelmann *et al.*, 2004) and leaf tissue sections (Yamori *et al.*, 2005). Ishikawa *et al.* (1995) also reported that the FDA staining method does not give an accurate estimation of cell survival in a large assembly of cells such as cross sections of intact tissues.

To solve many of these problems, the dye 5- (6-) sulfofluorescein diacetate (SFDA) appears to be effective. It was previously reported that all living cells and spores of microbial strains, including bacteria, actinomycetes, cellular slime molds and algae, are clearly stained with SFDA (Tsuji *et al.*, 1995). The basic principle is similar to that of FDA (Fig. 1). That is, non-fluorescent SFDA is converted to a fluorescent compound, fluorescein-5- (and 6-) sulfonic acid (FSA; mixed isomers), through the hydrolyzing ac-

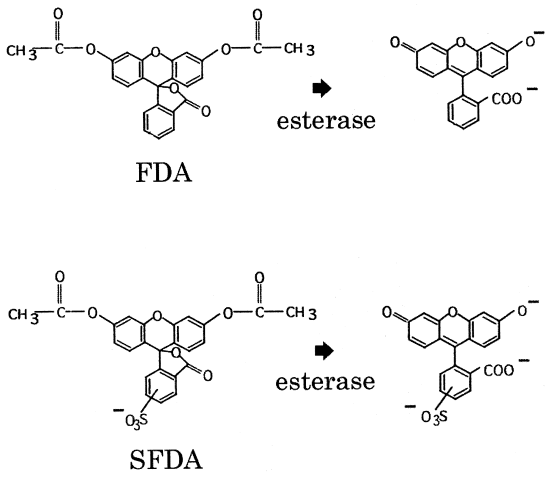

Fig. 1. Transformations of FDA and SFDA by esterase. Non-fluorescent FDA is converted to a fluorescent substance, fluorescein, by endogenous esterase activity. Non-fluorescent SFDA is also converted to a fluorescent compound, fluorescein-5- (and 6-) sulfonic acid (FSA; mixed isomers), by endogenous esterase activity.

tivity of esterase in the living cells. Ethanol (EtOH), a solvent of SFDA, penetrates cell walls and membranes, allowing SFDA to diffuse into the cytoplasm easily. Almost all living cells are stained by SFDA, and its ability to distinguish living from dead cells is superior to that of FDA (Tsuji *et al*., 1995).

In this paper, we propose a novel application of SFDA for the assessment of cell survival in leaf cross-sections of alpine plants using a confocal laser scan microscope (LSM). Alpine plants generally have the ability to tolerate freezing stress (Körner and Larcher, 1988; Yamori *et al*., 2005), and therefore, detection of differences between the results of a number of viability tests was thought to be relatively easy. This new procedure was also compared with two conventional methods, the FDA-staining method and electrolyte leakage test. In addition, we applied this method to assessment of the freezing tolerance of leaves of alpine plants.

Materials and methods

Plant materials

Plants of *Empetrum nigrum* L. var. *japonicum* K. Koch (Empetraceae), *Arcterica nana* (Maxim.) Makino, *Loiseleuria procumbens* L. Desvaux, *Phyllodoce nipponica* Makino, and *Vaccinium vitis-idaea* L. (Ericaceae) were dug out from the same west slope on Mt. Iwo (36°00'N, 138°22'N, 2760 m a.s.l.) in the Yatsugatake mountains, Nagano, Japan, in May 2001. The plant samples with soil were immediately wrapped with wet towels in plastic bags, kept on ice, and brought to the laboratory. The plants were then transferred to 1.3 *l* plastic pots and cultivated in an open place at Shizuoka University (34°57'N, 138°25'W, 55 m a.s.l.) where they were watered twice a week. The following examinations were conducted on 1 March 2002.

Freezing treatments

Freezing treatments were conducted according to previous studies with some modifications (Pennycooke *et al*., 2003; Shou *et al*., 2004; Yamori *et al*., 2005). The plants, including roots with soil, were kept in small polyethylene bags and cooled at a rate of 2.0 °C h^{-1} from 4 to −16°C in the dark in programmed freezers. Samples were removed when the temperature reached −5, −10 and −16°C, respectively. Some of the samples cooled to −16°C were then transferred to a freezer at −30°C for 7 h. Some of these samples were then transferred to −80°C for 25 h. All plants cooled to −5, −10, −16, −30 and −80°C were subsequently thawed in the dark at 4°C.

Fluorescence probes

SFDA (Molecular Probes, Inc., Eugene, OR, USA) was dissolved in EtOH at a final concentration of 2.0 mM. For the stock solution, FDA was dissolved in dimethyl sulfoxide (DMSO) at 10 mM. Just prior to use, the FDA in the DMSO solution was diluted to 0.23 mM with distilled water.

Determination of freezing tolerance

1. FDA- and SFDA-staining methods

Determination of freezing tolerance using the FDA-staining method was conducted

according to Yamori *et al*. (2005). The freezing tolerance of the plants was examined after 3 days of rewarming at 4°C in the dark. This duration was suggested as being the best condition for examining the freezing tolerance, because irreversible damage is clear and no significant regrowth has yet occurred (Mantyla *et al*., 1995). Tissue sections, with a thickness of approximately three cells, were cut with a new razor blade and placed in a micro-tube (1.5 m*l*) then soaked in 50 μl of 0.23 mM FDA or 2.0 mM SFDA solution for approximately 60 min at room temperature (from 20°C to 25°C). The sections were observed under a confocal laser scan microscope (LSM410, Zeiss, Oberkochen, Germany). From analyses of the concentration dependence of SFDA, we determined that 2.0 mM SFDA was optimal for evaluations of the freezing tolerance (data not shown). The excitation and emission wavelengths selected were 493 and 510 nm, respectively, and the degree of the cell survival was assessed. Only cells that exhibited a bright fluorescence from their cytosol were judged as viable.

The survival ratio (%) was calculated as the ratio of cells emitting fluorescence to total cells. The total cell number was counted in the same field of the microscope. In leaves of *V. vitis-idaea*, the total cell number counted using the microscope was approximately 200. At each freezing temperature, at least three leaf sections from different shoots were examined for each species.

2. Electrolyte leakage test

The electrolyte leakage test was conducted according to Yamori *et al*. (2005). Immediately after the frozen plants were thawed at 4°C, their leaves were removed and placed in screw-cap glass vials containing distilled water at a ratio of 0.1 g leaf sample (fresh weight): 10 m*l* of distilled water. The glass vials were kept at 4°C for 36 h in the dark. The conductivity of the supernatant was then measured with an electro-conductivity meter (Twin Compact Meter, Horiba, Kyoto, Japan). Each measurement was carried out in triplicate for each freezing temperature.

Results and discussion

We observed fluorescence images of cross-sections of unfrozen leaves of *V. vitis-idaea* using both the FDA- and SFDA-staining methods (Fig. 2). After freezing treatment at −80°C, no fluorescence was detected; the image was black (data not shown). In the unfrozen leaves, the FDA-staining method failed to stain all the cells (Fig. 2A), while the SFDA-staining method stained almost 100% (Fig. 2B). The same results were obtained with the other species examined (data not shown). The FDA-staining method, in particular, failed to stain the spongy tissue cells rather than the palisade tissue cells (Fig. 2A). Cell characteristics are known to differ between spongy and palisade parenchyma cells even within the same leaf (for a review, see Terashima, 1989; Terashima and Hikosaka, 1995), and since FDA does not efficiently penetrate some types of membrane (for a review, see Harding and Benson, 1995), low penetration of FDA might fail to stain the spongy tissue cells in particular.

After freezing treatment at −30°C and −80°C, the survival ratios resulting from both FDA- and SFDA-staining were almost 0% for leaves of *A. nana*, *E. nigrum*, *P. nipponica* and *V. vitis-idaea* (Fig. 3). Moreover, after freezing treatment at −80°C, the survival ratios

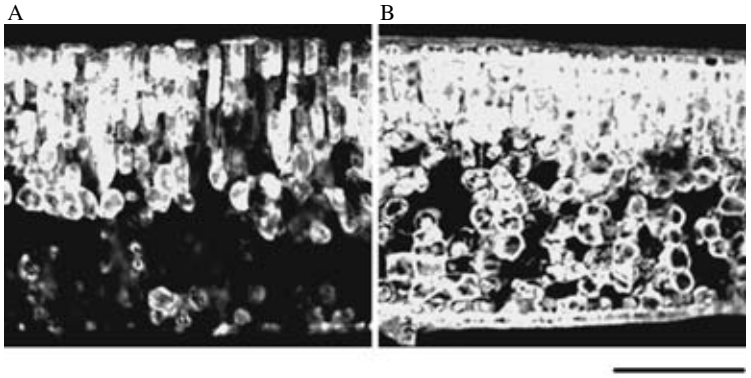

Fig. 2. Fluorescent images of cross-sections of unfrozen leaves of *V. vitis-ideaea* with the FDA-(A) and SFDA-staining methods (B). Bar=100 μm.

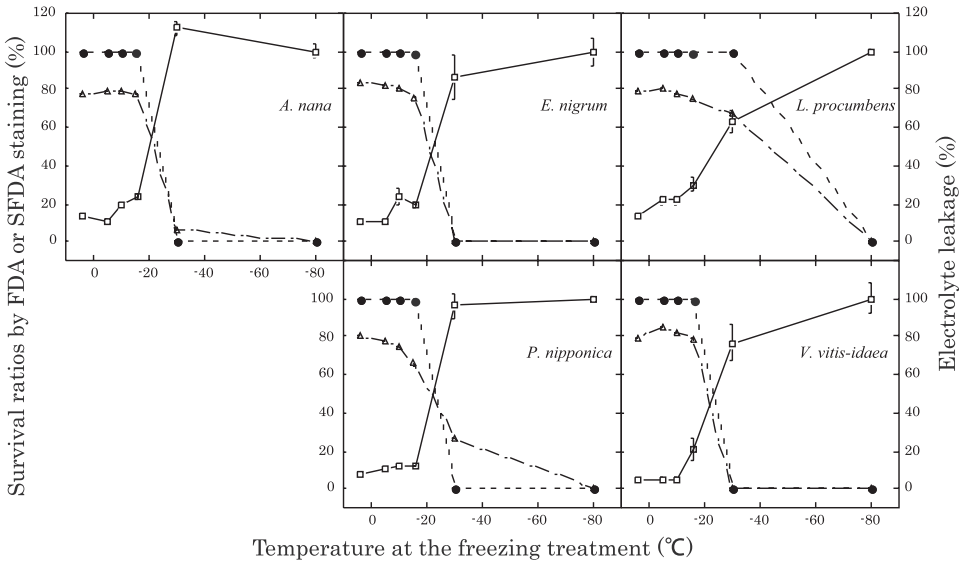

Fig. 3. Effects of freezing on the cell survival ratios (%) determined with the FDA- and SFDA-staining methods, and electrolyte leakage test (%) in five alpine plants. Values are means±SD (n=3). Open triangles: FDA-staining, closed circles: SFDA-staining, open squares: electrolyte leakage.

obtained by both staining methods were 0% for leaves of *L. procumbens*. On the other hand, after freezing treatments between −5°C and −16°C, leaves of *A. nana*, *E. nigrum*, *P. nipponica* and *V. vitis-idaea* were stained almost 100% by SFDA, but only 80% of the cells were stained by FDA (Fig. 3). The same tendencies were observed in leaves of *L. procumbens* after freezing treatments between −5°C and −30°C. The survival ratios in control samples of all species showed only 80% staining with the FDA-staining method,

as reported previously (Ogawa *et al.*, 1997; Steward *et al.*, 1999; Wang *et al.*, 2001; Winkelmann *et al.*, 2004; Yamori *et al.*, 2005). The low survival ratios with the FDA-staining method are thought to be due to the low penetration of FDA and its easy leakage from cells (Söderstöme, 1979; Thomas *et al.*, 1979; Harding and Benson, 1995). On the other hand, the SFDA-staining method was able to stain nearly all mesophyll cells in the leaves (Fig. 2). The combination of SFDA and EtOH is considered the most effective method of detecting living cells for the following reasons (Tsuji *et al.*, 1995): first, EtOH increases penetration of the cell membrane, greatly facilitating the penetration of SFDA into the cells. Second, the sulfonic group of fluorescein produced from SFDA may bind more strongly to positively charged cellular proteins compared to the fluorescein produced from FDA (Fig. 1).

The survival ratios (%) of leaf cells with the SFDA-staining method and electrolyte leakage (%), shown as relative electrolyte conductivity, are compared in Fig. 3. In this study, samples treated at −80°C were used to represent 100% injury for the electrolyte leakage test. Both methods indicated that most leaf cells in all samples survived freezing treatment at −16°C. For *V. vitis-idaea* treated at −16°C, the survival ratio with the SFDA-staining method was 100%, while with electrolyte leakage was 20.8%. For *L. procumbens* treated at −30°C, the SFDA-staining method indicated high survival ratios (100%), while the electrolyte leakage test showed substantial injuries (63.3%). These findings indicate that the electrolyte leakage test overestimates the freezing injuries. After freezing treatment at −30°C, the survival ratios of *A. nana*, *E. nigrum*, *P. nipponica*, and *V. vitis-idaea* decreased to almost 0% when assessed with the SFDA-staining method. It is clear that the results of the SFDA-staining method and electrolyte leakage test were similar in *A. nana* and *P. nipponica*, as electrolyte leakage in *A. nana* and *P. nipponica* increased to 112 and 96.0%, respectively, after freezing treatment at −30°C. However, electrolyte leakage in *E. nigrum* and *V. vitis-idaea* increased to only 86.7 and 76.5% after freezing at −30°C. These results indicate that the electrolyte leakage test underestimates freezing injury. Thus, there was considerable inconsistency between the SFDA-staining method and electrolyte leakage test. Yamori *et al.* (2005) observed that the values obtained with the electrolyte leakage test were generally correlated with the survival ratios obtained with the FDA-staining method in nine alpine plants. However, the electrolyte leakage test greatly overestimated the freezing injuries in three species. The same disadvantage has been frequently pointed out in other previous studies (Pellett and Carter, 1981; Burr *et al.*, 1990; James and Bert, 1990).

The electrolyte leakage test has been widely used for determining the freezing injury in viability tests. The test provides a simple, rapid and qualitative technique, and moreover, is generally correlated with the FDA- and SFDA-staining methods, although it sometimes overestimates or underestimates the survival ratios (Fig. 3). Therefore, we conclude that the electrolyte leakage test can be used for rough estimations of freezing tolerance. However, we recommend the use of the SFDA-staining method rather than the electrolyte leakage test when a more accurate and reliable analysis is needed; for example, in comparisons of differences in freezing tolerance between palisade and spongy tissue cells. The SFDA-staining method also offers the following advantages compared with the FDA-staining method: 1) it overcomes low penetration of the dye by using EtOH as a solvent and 2) its reaction with cellular proteins is higher as SFDA has a sulfonic group. These

findings show that the SFDA-staining method is more convenient, accurate and reproducible.

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References

Aronsson, A. and Eliasson, L. (1970): Frost hardiness in scots pine (*Pinus sylvestris* L.). I. Conditions for test on hardy plant tissues and for evaluation of injuries by conductivity measurements. Stud. For. Suec., **77**, 1–28.

Boorse, G.C., Ewers, F.W. and Davis, S.D. (1998): Response of chaparral shrubs to below-freezing temperatures: Acclimation, ecotypes, seedlings vs. adults. Am. J. Bot., **85**, 1224–1230.

Burke, J.J. (2001): Identification of genetic diversity and mutations in higher plant acquired thermotolerance. Physiol. Plant., **112**, 167–170.

Burr, K.E., Tinus, R.W., Walner, S.J. and King, R.M. (1990): Comparison of three cold hardiness tests for conifer seedlings. Tree Physiol., **6**, 351–369.

Chen, W.P., Li, P.H. and Chen, T.H.H. (2000): Glycinebetaine increases chilling tolerance and reduces chilling-induced lipid peroxidation in *Zea mays* L. Plant Cell Environ., **23**, 609–618.

Dexter, S.T. (1956): The evaluation of crop plants for winter hardiness. Adv. Agron., **8**, 203–239.

Dexter, S.T., Tottingham, W.E. and Graber, L.F. (1930): Preliminary results in measuring the hardiness of plants. Plant Physiol., **5**, 215–223.

Dexter, S.T., Tottingham, W.E. and Graber, L.F. (1932): Investigations of the hardiness of plants by measurement of electrical conductivity. Plant Physiol., **7**, 63–78.

Duncan, D.R. and Widholm, J.M. (1990): Measurement of viability suitable for plant tissue culture. Plant Cell and Tissue Culture, ed. by J.W. Pollard and J.M. Walker. Clilton, The Humana Press, 29–37 (Methods in Molecular Biology, Vol. 6).

Gaff, D.F. and Okong'Oogola, O. (1971): The use of non-permeating pigments for testing the survival of cells. J. Exp. Bot., **22**, 756–758.

Harding, K. and Benson, E.E. (1995): Biochemical and molecular methods for assessing damage, recovery and stability in cryopreserved plant germplasm. Genetic Preservation of Plant Cells in Vitro, ed. by B. Grout. New York, Springer, 113–169.

Hou, B.H. and Lin, C.G. (1996): Rapid optimization of electroporation conditions for soybean and tomato suspension cultured cells. Plant Physiol., **111** (Suppl. 2), 166.

Huang, C.N., Cornejo, M.J., Bush, D.S. and Jones, R.L. (1986): Estimating viability of plant protoplasts using double and single staining. Protoplasma, **135**, 80–87.

Hurry, V., Strand, A., Furbank, R. and Stitt, M. (2000): The role of inorganic phosphate in the development of freezing tolerance and the acclimatization of photosynthesis to low temperature is revealed by the *pho* mutants of *Arabidopsis thaliana*. Plant J., **24**, 383–396.

Iija, P. and Jiri, Z. (1998): The use of a conductivity measurement method for assessing freezing injury. I. Influence of leakage time, segment number, size, and shape, in a sample on evaluation of the degree of injury. Environ. Exp. Bot., **40**, 1–10.

Ishikawa, M., Robertson, A.J. and Gusta, L.V. (1990): Effect of temperature, light, nutrients and dehardening on abscisic acid induced cold hardiness in *Bromus inermis* Leyss suspension cultured cells. Plant Cell Physiol., **31**, 51–59.

Ishikawa, M., Robertson, A.J. and Gusta, L.V. (1995): Comparison of viability tests for assessing cross-adaptation to freezing, heat and salt stresses induced by abscisic acid in bromegrass (*Bromus inermis*

Leyss) suspension cultured cells. Plant Sci., **107**, 83–93.
James, B.C. and Bert, T.S. (1990): The distinction between living and dead plant tissue-viability tests in cold hardiness research. Cryobiology, **27**, 194–211.
Ketche, D.O., Buman, D.H. and Ballard, A.L. (1972): Relationship of electrolytic conductance to cold injury and acclimation in fruit trees. J. Am. Soc. Hortic. Sci., **97**, 403–406.
Körner, C.h. and Larcher, W. (1988): Plant life in cold climates. Symp. Soc. Exp. Biol., **42**, 25–57.
Mantyla, E., Lang, V. and Palva, E.T. (1995): Role of abscisic acid in drought-induced freezing tolerance, cold acclimation, and accumulation of LTI78 and RAB18 proteins in *Arabidopsis thaliana*. Plant Physiol., **107**, 141–148.
Matanguihan, R.M., Konstantinov, K.B. and Yoshida, T. (1994): Dielectric measurement to monitor the growth and the physiological states of biological cells. Bioprocess Eng., **11**, 213–222.
Mattson, A.M., Jensen, C.O. and Dutcher, R.A. (1947): Triphenyl tetrazolium chloride as a dye for vital tissues. Science, **106**, 294–295.
Menges, M. and Murray, J.A.H. (2004): Cryopreservation of transformed and wild-type Arabidopsis and tobacco cell suspension cultures. Plant J., **37**, 635–644.
Nash, D.T. and Davies, M.E. (1975): Isoenzyme changes during the growth cycle of Paul's scarlet rose cell suspensions. Phytochemistry, **14**, 2113–2118.
Neuner, G., Bannister, P. and Larcher, W. (1997): Ice formation and foliar frost resistance in attached and excised shoots from seedlings and adult trees of *Nothofagus menziesii*. N. Z. J. Bot., **35**, 221–227.
Nobel, P.S. and Barrera, E.D.L. (2003): Tolerances and acclimation to low and high temperatures for cladodes, fruits and roots of a widely cultivated cactus, *Opuntia ficus-indica*. New Phytol., **157**, 271–279.
Nobel, P.S., Wang, N., Balsamo, R.A., Loik, M.E. and Hawke, M.A. (1995): Low-temperature tolerance and acclimation of *Opuntia* spp. after injecting glucose or methylglucose. Int. J. Plant Sci., **156**, 496–504.
Noland, T.L. and Mohammed, G.H. (1997): Fluorescein diacetate as a viability stain for tree roots and seeds. New Forests, **14**, 221–232.
Ogawa, R., Ishikawa, M., Niwata, E. and Oosawa, K. (1997): Cryopreservation of shoot primordial cultures of melon using a slow prefreezing procedure. Plant Cell, Tissue and Organ Culture, **49**, 171–177.
Parker, J. (1953): Some applications and limitations of tetrazolium chloride. Science, **118**, 77–79.
Pellett, H.M. and Carter, J.V. (1981): Effect of nutritional factors on cold hardiness of plants. Horticultural Reviews, Vol. 3, ed. by J. Janick. Wesport, AVI Publ., 144–171.
Pennycooke, J.C., Jones, M.L. and Stushnoff, C. (2003): Down-regulation α-galactosidase enhances freezing tolerance in transgenic petunia. Plant Physiol., **133**, 901–909.
Pihakaski-Maunsbach, K., Tamminen, I., Pietiäinen, M. and Griffith, M. (2003): Antifreeze proteins are secreted by winter rye cells in suspension culture. Physiol. Plant., **118**, 390–398.
Pokorna, J., Schwarzerova, K., Zelenkova, S., Petrasek, J., Janotova, I., Capkova, V. and Opatrny, Z. (2004): Sites of action filament initiation and reorganization in cold-treated tobacco cells. Plant Cell Environ., **27**, 641–653.
Sakai, A. (1955): A method for testing the survival of the twig. Teion Kagaku, Seibutsu-hen (Low Temp. Sci., Ser. B), **13**, 43–50.
Shou, H., Bordallo, P., Fan, J.B., Yeakley, J.M., Bibikova, M.,Sheen, J. and Wang, K. (2004): Expression of an active tobacco mitogen-activated protein kinase kinase kinase enhances freezing tolerance in transgenic maize. Proc. Natl. Acad. Sci. USA, **101**, 3298–3303.
Smith, B.A., Reider, M.L. and Fletcher, J.S. (1982): Relationship between vital staining and subculture growth during the senescence of plant tissue culture. Plant Physiol., **70**, 1228–1230.
Söderstöme, B.E. (1979): Some problems in assessing the fluorescein diacetate-active fungal biomass in the soil. Soil Biol. Biochem., **9**, 59–63.
Steponkus, P.L. and Lanphear, F.O. (1967): Refinement of the triphenyl tetrazolium chloride method of determining cold injury. Plant Physiol., **42**, 1423–1426.
Sterigo, B.G. and Howell, G.S. (1973): Evaluation of viability tests for cold stressed plants. J. Am. Soc. Hortic. Sci., **98**, 325–330.
Steward, N., Martin, R., Engasser, J.M. and Goergen, J.L. (1999): A new methodology for plant cell viability assessment using intracellular esterase activity. Plant Cell Rep., **19**, 171–176.
Swain, D. and De, D.N. (1994): Vital staining, a technique for rapid screening of plant protoplast viability. Indian J. Exp. Biol., **32**, 501–506.

Terashima, I. (1989): Productive structure of a leaf. Photosynthesis, ed. by W.R. Briggs. New York, Alan R. Liss, 207–226.

Terashima, I. and Hikosaka, K. (1995): Comparative ecophysiology/anatomy of leaf and canopy photosynthesis. Plant Cell Environ., **18**, 1111–1128.

Thomas, J.A., Buchsbaum, R.N., Zimnial, A. and Racker, E. (1979): Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated *in situ*. Biochemistry, **18**, 2210–2218.

Towill, L.E. and Mazur, P. (1974): Studies on the reduction of 2, 3, 5-triphenyltetrazolium chloride as a viability assay for plant tissue cultures. Can. J. Bot., **53**, 1097–1102.

Tsuji, T., Kawasaki, Y., Takeshima, S., Sekiya, T. and Tanaka, S. (1995): A new fluorescence staining assay for visualizing living microorganisms in soil. Appl. Environ. Microbiol., **61**, 3415–3421.

Van den Driessche, R. (1976): Prediction of cold hardiness in douglass fir seedlings by index of injury and conductivity methods. Can. J. For. Res., **6**, 511–515.

Vaňková, R., Kuncová, G., Opatrná, J., Süssenbeková, H., Gaudinová, A. and Vaněk, T. (2001): Two-dimensional fluorescence spectroscopy—a new tool for the determination of plant cell viability. Plant Cell Rep., **20**, 41–47.

Wang, C.Y., Bowen, J.H., Weir, I.E., Allan, A.C. and Ferguson, I.B. (2001): Heat-induced protection against death of suspension-cultured apple fruit cells exposed to low temperature. Plant Cell Environ., **24**, 1199–1207.

Wang, Q., Mawassi, M., Sahar, N., Li, P., Violeta, C.T., Gafny, R., Sela, I., Tanne, E. and Perl, A. (2004): Cryopreservation of grapevine (*Vitis* spp.) embryogenic cell suspensions by encapsulation-vitrification. Plant Cell, Tissue and Organ Culture, **77**, 267–275.

Widholm, J.M. (1972): The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol., **47**, 189–194.

Winkelmann, T., Mußmann, V. and Serek, M. (2004): Cryopreservation of embryogenic suspension cultures of *Cyclamen persicum* Mill. Plant Cell Rep., **23**, 1–8.

Yamori, W., Kogami, H. and Masuzawa, T. (2005): Freezing tolerance in alpine plants as assessed by the FDA-staining method. Polar Biosci., **18**, 73–81.